

AD \_\_\_\_\_

Award Number: DAMD17-01-1-0563

TITLE: Role of APAf-1Caspase-9 Pathway in Breast Tumor  
Development

PRINCIPAL INVESTIGATOR: Gabriel Nunez, M.D.

CONTRACTING ORGANIZATION: University of Michigan  
Ann Arbor, Michigan 48109-1274

REPORT DATE: September 2002

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command  
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;  
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

20030328 272

**REPORT DOCUMENTATION PAGE**Form Approved  
OMB No. 074-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503

<b>1. AGENCY USE ONLY (Leave blank)</b>		<b>2. REPORT DATE</b> September 2002	<b>3. REPORT TYPE AND DATES COVERED</b> Annual (15 Aug 01 - 14 Aug 02)	
<b>4. TITLE AND SUBTITLE</b> Role of APAf-1Caspase-9 Pathway in Breast Tumor Development			<b>5. FUNDING NUMBERS</b> DAMD17-01-1-0563	
<b>6. AUTHOR(S) :</b> Gabriel Nunez, M.D.				
<b>7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)</b> University of Michigan Ann Arbor, Michigan 48109-1274  E-Mail:bclx@umich.edu			<b>8. PERFORMING ORGANIZATION REPORT NUMBER</b>	
<b>9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)</b> U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012			<b>10. SPONSORING / MONITORING AGENCY REPORT NUMBER</b>	
<b>11. SUPPLEMENTARY NOTES</b>				
<b>12a. DISTRIBUTION / AVAILABILITY STATEMENT</b> Approved for Public Release; Distribution Unlimited			<b>12b. DISTRIBUTION CODE</b>	
<b>13. ABSTRACT (Maximum 200 Words)</b>  The Aim of the project was to assess the function of Apaf-1/Caspase-9 in tumorigenesis in the mouse. We panned to use mutant mice expressing a conditional knockout (KO) Apaf-1 gene in the breast as the standard Apaf-1 KO mutation is embryonic lethal. Unfortunately, the generation of these mutant mice proved to be technically difficult and the production of the mutant mice has been delayed. Due to these problems, we requested a 1 year no-cost extension of the grant which was granted. As an alternative approach, we have obtained FOG mutant mice from Jackson laboratory which exhibit a hypomorphic Apaf-1 allele. These mutant mice are viable and lack detectable expression of Apaf-1 protein and the Apaf-1 transcripts are 10-20 fold less abundant than those in wild-type mice. We have developed a system to express stably small (21-29 bp) interfering RNAs (siRNAs) targeted against Apaf-1 transcripts. Eight hairpin oligonucleotides were designed to target four different regions of the Apaf-1 transcript. We constructed and tested multiples constructs and identified a RNAi construct that effectively inhibit the expression of Apaf-1 protein in breast cancer cells.				
<b>14. SUBJECT TERMS</b> breast, research, breast cancer			<b>15. NUMBER OF PAGES</b> 8	
			<b>16. PRICE CODE</b>	
<b>17. SECURITY CLASSIFICATION OF REPORT</b> Unclassified	<b>18. SECURITY CLASSIFICATION OF THIS PAGE</b> Unclassified	<b>19. SECURITY CLASSIFICATION OF ABSTRACT</b> Unclassified	<b>20. LIMITATION OF ABSTRACT</b> Unlimited	

## Table of Contents

Cover.....	1
SF 298.....	2
Table of Contents.....	3
Introduction.....	4
Body.....	5-7
Key Research Accomplishments.....	8
Reportable Outcomes.....	8
Conclusions.....	8
References.....	8
Appendices.....	None

## INTRODUCTION

Recent evidence indicates that alterations of the cell death pathway contribute to the pathogenesis of many diseases including cancer and may play a role in resistance to therapy (1). However, the contribution of specific pathways of apoptosis to cancer development is poorly understood and the role that apoptosis plays in the resistance of tumors to therapy is highly controversial. A major pathway of apoptosis links intracellular death stimuli to the activation of caspase-9 through Apaf-1 (2). It has been shown that transformed mouse fibroblasts deficient in Apaf-1 exhibit increased tumorigenicity and are less susceptible to several apoptotic stimuli (3). Furthermore, There is evidence that Apaf-1 expression is important for melanoma development (4) and low level Apaf-1 activity has been found in ovarian cancer (5-6). However, direct evidence that the Apaf-1/caspase-9 pathway is important for tumor developent is lacking. Mice deficient in Apaf-1 die during development or shortly after birth (7), precluding their use in models of cancer development. Our hypothesis is that the endogenous Apaf-1/caspase-9 pathway suppresses tumor development through the activation of apoptosis in cancer-prone epithelial cells in the breast. To test this hypothesis, we propose to study mutant mice expressing a hypomorphic allele of Apaf-1.

## BODY

This is the annual report for a project funded for 1 year entitled "Role of Apaf-1/Caspase-9 pathway in Breast Tumor Development. The Aim of the project was to assess the function of Apaf-1/Caspase-9 in tumorigenesis in the mouse. Initially we proposed to generate transgenic mice in which dominant negative forms of Apaf-1 or Caspase-9 are expressed in the breast. A potential problem with this approach is that dominant negative forms need to be expressed at high levels relative to those of the endogenous proteins for inhibitory activity. To avoid this problem, we decided to use mutant mice expressing a conditional knockout (KO) Apaf-1 gene in the breast as the standard Apaf-1 KO mutation is embryonic lethal. Dr. Francesco Cecconi who initially generated Apaf-1 KO mice (3) agreed to provide the conditional KO mice for these experiments. Unfortunately, the generation of these mutant mice proved to be technically difficult and the production of the mutant mice has been delayed. Due to these problems, we requested a 1 year no-cost extension of the grant which was granted. As an alternative approach, we have obtained FOG mutant mice from Jackson laboratory which exhibit a hypomorphic Apaf-1 allele. These mutant mice lack detectable expression of Apaf-1 protein and the Apaf-1 transcripts are 10-20 fold less abundant than those in wild-type mice (8). Most FOG mutant mice are viable (8) and therefore they can be used to assess the role of Apaf-1 in tumor development in vivo. We plan during the next year to expand the colony of FOG mutant and to begin to analyze the susceptibility of FOG mice to tumor development as they compare with FOG +/- and wild-type mice.

In addition during the last year, we have developed a system to express stably small (21-29 bp) interfering RNAs (siRNAs) targeted against Apaf-1 transcripts. RNA interference (RNAi) is the process of sequence-specific gene silencing in animal and plants initiated by double-stranded RNA that results in the enzymatic degradation of complementary RNA (9-11). Eight hairpin oligonucleotides were designed to target four different regions of the Apaf-1 transcript. The sequence of the sense (s) and antisense (as) oligonucleotides are shown below. The ability of the optimal candidates were tested by transient co-transfection of the retroviral plasmid and a construct expressing Apaf-1.

1. Apaf-1 RNAi 1s 5' CTA GAC CGT CGA CGG ACA TCA AGA CCT ACT TCG GTA GGA TGT CTT GAT GTC CTT TTT CTA GAG CTC AAC 3'
2. Apaf-1 RNAi 1as 5' GTT GAG CTC TAG AAA AAG GAC ATC AAG ACA TCC TAC CGA AGT AGG ATG TCT TGA TGT CCG TCG ACG GTC TAG 3'
3. Apaf-1 RNAi 2s 5' GTA GAC CGT CGA CCC TAC GTA TCA TTC TAC AAT TCG TTG TAG AAT GAT ACG TAG GTT TTT CTA GAG CTC AAC 3'
4. Apaf-1- RNAi 2as 5' GTT GAG CTC TAG AAA AAC CTA CGT ATC ATT CTA CAA CGA ATT GTA GAA TGA TAC GTA GGG TCG ACG GTC TAG 3'

5. Apaf-1 RNAi 3s 5' CTA GAC CGT CGA CGG TCA CCA TAC ATG GAA TGT  
TCG CAT TCC ATG TAT GGT GAC CTT TTT CTA GAG CTC AAC 3'

6. Apaf-1 RNAi3as 5' GTT GAG CTC TAG AAA AAG GTC ACC ATA CAT GGA  
ATG CGA ACA TTC CAT GTA TGG TGA CCG TCG ACG GTC TAG 3'

7. Apaf-1 s(H1) 5' GAT CCC CGG ACA TCA AGA CAT CCT ACT TCA AGA GAG  
TAG GAT GTC TTG ATG TCC TTT TTG G AAA 3'

8. Apaf-1as(H1) 5' AGC TTT TCC AAA AAG GAC ATC AAG ACA TCC TAC TCT  
CTT GAA GTA GGA TGT CTT GAT GTC CGG G 3'

The sense and antisense oligonucleotides for each region were incubated annealing buffer (10nM TRIS-HCl pH 7.5, 100mM NaCl, 1mM EDTA pH 8.0), digested with SalI and XbaI, and ligated into three different expression plasmids. These included pAV U6+27 (a gift of Dr. Engelke, the University of Michigan), pMND-Banshee (a gift of John Rossi, University of California, City of Hope and pSUPER (9-11). These plasmids use different promoters and regulatory sequences and we wished to compare their abilities to inhibit Apaf-1 expression. We transfected these expression and control plasmids into human embryonic HEK293T cells, A2780 and MCF-7 cancer cells. Immunoblotting analysis with anti-Apaf-1 antibody revealed that the plasmid expressing RNAi oligonucleotides # 7 and # 8 was particularly effective in inhibiting Apaf-1 protein in HEK293T cells as determined in transient assays (Fig. 1). We also transfected the same construct into A2780 and MCF7 cells and selected cell clones. Immunoblotting analysis revealed several cell clones which were deficient in Apaf-1 when compared to parental cells (Fig. 2). We plan to use this construct to study the role of Apaf-1 in the regulation of cancer cell growth and response to chemotherapeutic drugs.

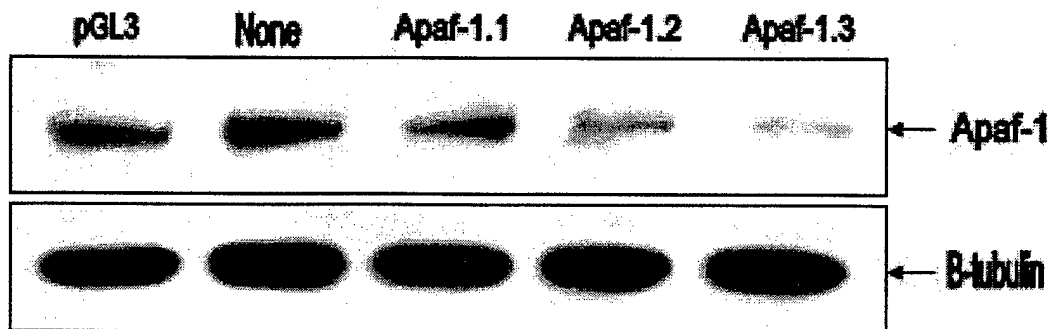


FIGURE 1. Inhibition of Apaf-1 protein expression by RNAi constructs. HEK293T cells were transiently transfected with control plasmid (pGL2), no plasmid (None) or three plasmids expressing hairpin RNAi oligonucleotides Apaf-1.1-3. 48 hrs after transfection, cellular extracts were immunoblotted with monoclonal anti-Apaf-1 antibody. Loading control is shown below.

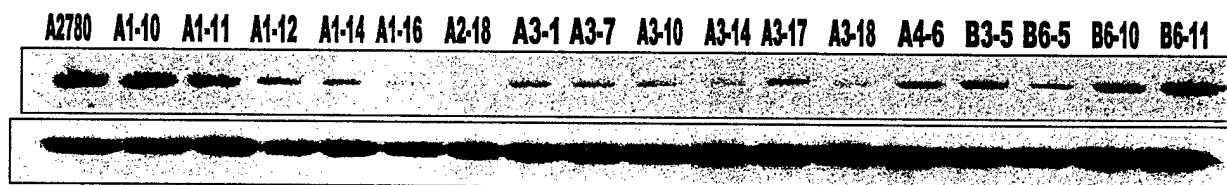


FIGURE 2. Inhibition of Apaf-1 protein expression by RNAi construct Apaf-1.3. A2780 cells were transfected with plasmid expressing hairpin RNAi oligonucleotide Apaf-1.3. Cell clones were isolated and cellular extracts from individual clones were immunoblotted with monoclonal anti-Apaf-1 antibody. Several clones are deficient in Apaf-1 including A1-16 and A2-18. Immunoblotting with anti- $\beta$ -tubulin (loading control is shown in lower panel).

## KEY RESEARCH ACCOMPLISHMENTS:

- Development of a RNAi construct to inhibit expression of Apaf-1

REPORTABLE OUTCOMES: None

## CONCLUSIONS:

We have developed a system to express stably small (21-29 bp) interfering RNAs (siRNAs) targeted against Apaf-1 transcripts. Eight hairpin oligonucleotides were designed to target four different regions of the Apaf-1 transcript. We have identified a construct that effectively inhibit the expression of Apaf-1 in breast cancer cells.

## REFERENCES:

1. R. W. Johnstone, A. Astrid. A. Ruefli, S. W. Lowe, *Cell* **108**: 153 (2002).
2. F. Cecconi , P. Gruss P, *Cell Mol Life Sci* **58**:1688 (2001).
3. F. Cecconi F, G. Alvarez-Bolado, B. I. Meyer, K. A. Roth, P Gruss, *Cell* **94**:727 (1998).
4. M. S. Soengas *et al.* *Science* **284**:156 (1999).
5. M. S. Soengas *et al.* *Nature* **409**:207 (2001).
6. J. R. Liu *et al.* *Cancer Res.* **62**:924 (2002).
7. B. B. Wolf *et al.* *J Biol Chem.* **276**:34244 (2001).
- 4:  
N. Honarpour, S. L. Gilbert, B. T. Lahn, X. Wang, J. Herz J, *Proc Natl Acad Sci U S A.* **98**:9683 (2001).
9. C. P. Paul, P. D. Good, I. Winer, D. R. Engelke. *Nat Biotechnol.* **20**:505 (2002).
10. N. S. Lee *et al.* *Nat Biotechnol* **20**:500 (2002).
11. T. R. Brummelkamp, R. Bernards, R. Agami, *Science* **296**: 550 (2002).